

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title: The potential hepatotoxicity of simultaneous exposure to inorganic Arsenic and Microcystin-LR in mice fed a high-fat diet

LAPR Number: 18-04-001

Principal Investigator: Exemption 6

Author of this Document: Exemption 6/RTP/USEPA/US

Date Originated: 03/23/2015

LAPR Expiration Date: 04/30/2018

Agenda Date: 04/08/2015

Date Approved: 04/16/2015

Date Closed: 03/06/2018

APPROVALS

| APPROVER | NAME | APPROVAL DATE | COMMENTS | |
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Administrative Information

1. Project Title (no abbreviations, include species):

The potential hepatotoxicity of simultaneous exposure to inorganic Arsenic and Microcystin-LR in mice fed a high-fat diet

Is this a continuing study with a previously approved LAPR?

No

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.

SSWR 2.3c Cyanobacteria, nutrients and land use - a nexus for sustainable water resources and human health protection.

b. What is the Quality Assurance Project Plan (QAPP) covering this project?

IRP-NHEERL-RTP/TAD/DTB/Exempt-2012-01-r01

3. EPA Principal Investigator/Responsible Employee:

| | | | |
|--|--|------------------------|------------------------|
| Principal Investigator Exemption 6 | Phone Number Exemption 6 | Division TAD | Mail Drop MD |
| | Lotus Notes Address Exemption 6 Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/ US | Branch DTB | |

4. Alternate Contact:

| | | | |
|---|--|------------------------|------------------------|
| Alternate Contact Exemption 6 | Phone Number Exemption 6 | Division TAD | Mail Drop MD |
| | Lotus Notes Address Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6 TP/USEPA/US | Branch DTB | |

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the

animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

This LAPR is designed to investigate the hepatotoxic effects of co-exposures to arsenic and the algal toxin, microcystin-LR (M-LR) in mice that are being fed a high-fat diet. Both toxicants are ubiquitous in nature, potent liver toxicants, and common food and drinking water contaminants. The possibility that their toxicities interact, and that this interaction may be enhanced by lifestyle factors such as dietary fat content, poses a potential threat to public health.

Arsenic is a naturally occurring metalloid that readily penetrates groundwater used for agriculture and drinking supplies. Dietary exposure to inorganic arsenic via contaminated drinking water and food products has significant health effects, especially in regions where arsenic is particularly abundant. The liver has been shown to be one of the primary targets of arsenic toxicity in both in-vitro and in-vivo models. M-LR is a potent hepatotoxic alkaloid secreted by cyanobacteria (blue-green algae). Human exposure to M-LR occurs primarily during Harmful Algal Bloom (HAB) events via recreation or consumption of contaminated food or drinking products. The increasing prevalence of these two naturally occurring toxicants in the environment may pose a threat to public health if their toxicities interact.

According to the two-hit hypothesis of liver disease (Day et al. 1998), chronic exposure to a hepatotoxic agent contributes to fatty liver development, making the liver susceptible to a “second hit” that stimulates progression to severe liver injury. Several factors have been shown to mediate this progression of simple steatosis (fatty liver) to steatohepatitis (fatty liver, inflammation, and cell death). The purpose of this study is to test the hypothesis that chronic subhepatotoxic arsenic exposure in the diet may sensitize the liver to an acute M-LR exposure associated with a HAB event, leading to significant liver injury. Additionally, it will investigate the affect of dietary fat content on this relationship. Diet-environmental interactions are known to play an important role in human disease, particularly in the development and progression of fatty liver disease. Studies have shown that chronic subhepatotoxic exposure to arsenic enhances hepatic injury caused by high-fat diet in mice, but there are no studies examining this interaction in the presence of another potent hepatotoxin such as M-LR.

The work proposed in this LAPR is important because both arsenic and M-LR are naturally occurring toxicants causing hepatotoxicity and having a common route of human exposure. Low-level exposures to inorganic arsenic in drinking water are common in places like the United States, as are intermittent exposures to M-LR that are occurring with the increasing frequency of HAB events in recent years. The possibility that these two toxicants can interact constitutes a data gap requiring the type of study proposed here. Furthermore, lifestyle factors such as dietary fat content should be examined under this context since obesity is correlated with increased incidence of liver disease.

Dose-finding studies will be conducted for M-LR sequentially in order to minimize instances of severe toxicity. For the main study, doses will be selected to produce marginal, if any, overt toxicity. The main endpoints of interest are histopathological and serum chemistry changes, rather than overt toxicity.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

There is no computer model or in-vitro bioassay system that can accurately predict the adverse effects of exposure to toxicants like M-LR, whose targets and mechanisms of toxicity are highly debated. The toxic manifestations of M-LR in people and laboratory animals are extremely variable and appear to involve a wide variety of tissues and unknown metabolic pathways. Additionally, the simultaneous administration of a toxin with immense documented species sensitivity like arsenic in an in-vitro system may bear little or no resemblance to the toxicity seen in an in-vivo system. The intact animal is necessary to encompass all potential mechanisms of interactions between the toxicants and diet.

b. Justify the species requested:

Several factors support our use of the mouse in the proposed research. The physiology of the mouse is well characterized, including a considerable knowledge of the structure and function of the liver (our focus). Many studies have examined key elements of this proposal in mice, including the toxicity of inorganic arsenic, or M-LR, and hepatic effects of a high-fat diet. Additionally, the past work of both **Exemption 6** has regularly utilized the mouse model in studies involving dosing regimens and experimental designs covered by this LAPR.

3. How was it determined that this study is not unnecessary duplication?

We have searched the literature listed in PubMed from 1980 to the present using keywords “Microcystin-LR,” “arsenic,” and “toxicity.” This literature search shows that there are no studies that have investigated the potential of M-LR and arsenic to interact.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

Dose-Finding Study:

Dose-finding studies will have to be conducted for M-LR because the literature lacks appropriate dose-response data using the proposed exposure regimen in 4-week old female mice. Initial doses for these dose-finding studies will be based on the reported oral LD50 and lowest observed adverse effect level (LOAEL) values in mice (Fawell et al. 1994). We will use initial doses of 0, 0.5, and 3.0 mg/kg, although we have requested sufficient animals to perform four follow-up dose-finding studies at higher or lower doses in order to determine appropriate doses for the main study. The appropriate dose will be defined as one that induces changes in serum endpoints (e.g., increased levels of ALT, SDH, AST and/or GGT) but no signs of overt toxicity (e.g., weight loss, changes in appearance). Doses will be administered via a single oral gavage, and animals will be sacrificed 24 hours post-dosing. Each dose-finding experiment will consist of two groups (treatment and control), with three mice per treatment group and two mice per control group.

Main Study:

The experimental design of this proposal is an attempt to expose animals to a similar regimen as that which occurs in human populations: exposure to chronic low-levels of arsenic in drinking water supplemented by a high-fat diet, with acute M-LR exposure from a HAB event. The definitive study would involve exposing 4-week old female mice on a high-fat diet to inorganic arsenic (as sodium arsenite) in drinking water for 30 days at a level of 5ppm of arsenic calculated as elemental arsenic, and then dosing with M-LR by oral gavage on day 30 of the exposure period. There will therefore be eight groups of animals (21 animals per group) given drinking water with or without arsenic and fed either a high-fat (40% calories from fat) or control (15% calories from fat) diet for 30 days, that will receive a single oral gavage on day 30 of either M-LR or sterile water as follows:

- Group 1: Control diet, water gavage
- Group 2: High-fat diet, water gavage
- Group 3: Control diet + arsenic in drinking water, water gavage
- Group 4: High-fat diet + arsenic in drinking water, water gavage
- Group 5: Control diet, M-LR gavage
- Group 6: High-fat diet, M-LR gavage
- Group 7: Control diet + arsenic in drinking water, M-LR gavage
- Group 8: High-fat diet + arsenic in drinking water, M-LR gavage

Treatment drinking water will be supplemented with 5 parts per million (ppm) sodium arsenite using conditioned water from the animal facility. This dose was chosen because it has been shown to have no effects on the livers of mice fed a low-fat control diet (Shi et al. 2014; Tan et al. 2011). AIN-93G purified rodent diet (containing no background arsenic) enriched with 40% calories from fat will be used as the treatment diet (Dyets, Bethlehem, PA). The AIN-93G diet is formulated to meet the nutritional and caloric needs of growing mice (Reeves et al. 1993) and without fat addition will be used as the control diet. M-LR doses will be based on the results of the dose-finding study. The selected dose will be expected to cause marginal, if any, overt toxicity when administered alone. This will maximize our ability to detect additive or synergistic effects of the M-LR+As+high-fat diet combination. M-LR dosing will be by single oral gavage in 0.1ml/mouse of dosing solution in sterile water.

In order to understand both the physiologic and metabolic effects of co-exposure to arsenic and M-LR on a high-fat diet, animals will be housed in standard metabolism cages. 4-week old female C57BL/6 mice received from Charles River Laboratories will be housed in groups of 3 mice per cage for 35 days (5 day acclimation period + 30 day exposure period). During the 5-day acclimation period, mice will be housed in groups of 3 in regular animal facility housing under standard environmental conditions with free access to rodent food and drinking water free of arsenic. From days 1 to 28 of the exposure period, mice will continue to be housed in the same groups of 3 in standard housing, but with free access to high- or low-fat rodent food

and drinking water supplemented with or without arsenic. On day 29, each group of three mice housed together in standard housing will be transferred en group to a metabolism cage that separates urine and feces for 48 hours (Nalgene, Rochester, NY). Urine will be collected from each metabolism cage on days 29 and 30 using standardized techniques developed and validated by the ^{Exemption 6} ^{Exemption 6} Lab. Mice will continue to have free access to the test diet and drinking water. On the morning of day 30 of the exposure period, animals will receive a single oral gavage of either sterile water or M-LR (at the dose determined appropriate from the dose-finding studies). Animals will be sacrificed 24-hours post-oral gavage, and will be weighed and necropsied for signs of M-LR- or arsenic-induced toxicity. Blood will be collected for serum analysis of endpoints indicative of hepatic toxicity. Liver will be weighed and tissues preserved for histological analysis.

Data on daily water consumption will be used to calculate total intake of arsenic. Daily water consumption for each cage is calculated as the difference between the weights of the water bottle each morning. Water bottles will be changed 2 to 3 times per week to minimize arsenite oxidation.

Neither food nor water intake has shown to be adversely affected by introduction into metabolism cages. Additionally, in our experience, there is no overt evidence of toxicity in any mice exposed to arsenic by consumption of treated drinking water at this dosage. Because we are aware of the need for consistent oversight of the well-being of mice during the exposure period, study personnel are solely responsible for the provision of food and water to mice, for the collection of urine and feces samples, and for the inspection of mice. Animals will be weighed 2 to 3 times per week and monitored daily during the week unless more observations are warranted because of adverse effects (see Sections 5f and 6e for details).

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

Dose-Finding Studies:

Each M-LR dose-finding experiment will consist of 2 treatment groups – treated and control. Three mice per treatment group and two mice per control group are the smallest sample sizes acceptable to provide meaningful information toward selecting appropriate dose levels. For the dose-finding studies we are therefore asking for a total of 25 mice (15 treated and 10 controls) for 5 possible dose-finding studies for M-LR, each study with 3 M-LR and 2 control animals.

Main Study:

The definitive study will comprise eight treatment groups, as outlined in Section B1, with a total of 18 female mice per group (and additional 3/group in case there are errors in the study that require an additional group). A sample size of 21 mice per group has been shown to be necessary to obtain statistical significance in group comparisons when utilizing metabolism cages such as those used here.

8 groups x 21 mice/group = 168 mice (84 Category C, 84 Category E). All animals exposed to M-LR will be considered Category E and all remaining animals will be considered Category C.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

| Categories | Adults | Offspring |
|--|--------|-----------|
| C) Minimal, transient, or no pain/distress: | 94 | 0 |
| D) Potential pain/distress relieved by appropriate measures: | 0 | 0 |
| E) Unrelieved pain/distress: | 99 | 0 |

4. Does this LAPR include any of the following:

- ☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

Dose-finding: Control animals will be dosed by single oral gavage with sterile water (0.1ml/mouse).

Main Study: Control animals will be fed either a control or high-fat diet supplemented with or without 5ppm arsenic in drinking water for 30 days. On day 30, they will be dosed by single oral gavage with sterile water

(0.1mL/mouse).

b. Survival Blood Collections (method, volume, frequency):

na

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

na

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

na

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

na

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

Animals will be housed 3/cage - all cages will be numbered and all animals will receive ear punches. The combination of cage number and ear punches will allow identification of individual mice for the duration of the study. Animals will be weighed 2 to 3 times a week and observed daily (in the morning, 9-10am) by study personnel.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

Dose-finding: Treated animals will be dosed by single oral gavage of M-LR (dosage between 0.5 and 3.0 mg/kg) in 0.1ml dosing solution in sterile water for treated animals, or sterile water alone for controls.

Main Study: Treated animals will be fed either a control or high-fat diet with or without 5ppm arsenic in drinking water for 30 days. On day 30, half the animals in all four groups will receive a single oral gavage of M-LR (dose determined from dose-finding studies) in 0.1mL/mouse dosing solution in sterile water, or sterile water alone. The summary of the different groups is:

Group 1: Control diet, water gavage (Category C)

Group 2: High-fat diet, water gavage (Category C)

Group 3: Control diet + arsenic, water gavage (Category C)

Group 4: High-fat diet + arsenic, water gavage (Category C)

Group 5: Control diet, M-LR gavage (Category E)

Group 6: High-fat diet, M-LR gavage (Category E)

Group 7: Control diet + arsenic, M-LR gavage (Category E)

Group 8: High-fat diet + arsenic, M-LR gavage (Category E)

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

na

c. Testing methods:

na

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

na

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

In the dose-finding study, animals will be monitored an hour after dosing in the morning and two subsequent times during early afternoon (approximately 1-2pm) and at the end of the work day (approximately 5-6pm).

During the main study treatment-related deaths are not expected. The most likely compound-induced effects expected will include alterations in food and/or water intake; changes in either serum chemistry endpoints (homeostasis measurements including serum protein and glucose levels; and/or enzyme levels that indicate hepatotoxicity effects) and/or histological effects in the liver that are indicative of hepatic toxicity. The dose levels used should produce few or no overt effects – in this manner, we will be able to

confidently identify additive or synergistic effects of simultaneous arsenic and M-LR. Nevertheless, repeated dose-finding studies, selection of marginally toxic doses in the main study, and frequent monitoring will be employed to minimize the chances of overt toxicity.

Animals will be weighed 2 to 3 times per week and observed daily in the morning (approximately 9-10am) during the week by study personnel. If treatment effects warrant additional monitoring, the number of times that animals are observed will be increased during both weekdays and weekends.

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

Analgesics will not be used because the interaction of analgesics with M-LR is unknown. The necessary study comparing treated animals with and without analgesics would actually lead to the use and eventual euthanasia of another set of animals. Also, the response to toxicity involves initiation of the stress cascade that, in itself, alters numerous normal responses to xenobiotics – the absence of the stress response would therefore create a situation that would make extrapolation to human or other animal populations more difficult.

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

No treatment-related deaths are expected.

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

na

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

na

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

na

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

na

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

☐ Yes ☒ No

f. Identify any surgical procedures performed at other institutions or by vendors:

na

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Animals will be monitored daily as detailed above in Sections 5f and 6e. The study personnel will be responsible for monitoring. We do not expect to see substantial overt toxicity due to either compound, but if such effects do occur (e.g. if there is a synergistic effect of M-LR and arsenic) the Attending Veterinarian will be notified immediately. Signs of hepatic toxicity can include reduced intake of food and concomitant loss of weight; lack of reactivity, lack of grooming and piloerection.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Any animals that require euthanasia due to non-responsiveness to interaction, inappetence, lethargy, hypothermia, diarrhea or weight loss greater than 10%, will be removed from the study; necropsies will be performed and blood and tissues will be collected for analysis as needed. Many of the arsenic changes seen in humans have been internal (e.g. fatty liver) and the doses we are using have been shown to induce only these changes in mice exposed to arsenic at these levels, and we therefore do not expect to see overt signs of toxicity.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

An extensive literature search using the keywords, "microcystin" and "analgesics" did not identify any studies in which analgesics had been used in conjunction with microcystin-LR. Analgesics will not be used, therefore, because the interaction of analgesics with M-LR is unknown. The necessary study comparing treated animals with and without analgesics would actually lead to the use and eventual euthanasia of another set of animals. Also, the response to toxicity involves initiation of the stress cascade that, in itself, alters numerous normal responses to xenobiotics - the absence of the stress response would therefore create a situation that would make extrapolation to human or other animal populations more difficult.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

| | |
|---|-----|
| a. Animals to be purchased from a Vendor for this study: | 193 |
| b. Animals to be transferred from another LAPR: LAPR Number that is the source of this transfer: | 0 |
| c. Animals to be transferred from another source: | 0 |
| d. Offspring produced onsite (used for data collection and/or weaned): | 0 |
| e. TOTAL NUMBER of animals for duration of the LAPR | 193 |

2. Species (limited to one per LAPR): Mouse/Mice

3. Strain: C57BL/6 mouse/mice

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

na

4. Sources of animals:
Charles River Laboratories, Raleigh

5. Provide room numbers where various procedures will be performed on animals:

Exem
Exem
Exem

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

no

Room Numbers:

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

none

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

For the first 28 days of the exposure period, mice will be housed three per cage in standard group cages with bedding. Mice will be housed three per cage in metabolism cages during the last 48 hours of the

exposure period.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

na

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

During standard group cage housing for days 1-28 of the exposure period, mice will be provided with a Enviro Dri and a polycarbonate hiding box for environmental enrichment. Housing in metabolism cages during the last 2 days of the exposure period precludes the presence of environmental enrichment articles.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.

Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

M-LR Maximum dose 3mg/kg oral LD50 is 5mg/kg in mice

Arsenic Maximum dose 0.6mg/kg/day arsenic given as sodium arsenite oral LD50 is 45mg/kg in mice

Chemical Alert Tags will be posted on all cages containing animals exposed to arsenic.

Appropriate signage will be posted notifying personnel that standard PPE will be sufficient for handling arsenic-treated animals and material; and that waste water will be handled as hazardous waste.

The HSRP for M-LR is "HSRP-164; "Potential adverse effects of algal toxins" ." We will not know what the maximum dose levels of M-LR will be until dose finding studies are completed. It should be understood that the chief problem we face is lack of toxicity and the maximum dose will depend on finding some effect – probably in serum changes and/or organ/body weight ratio alterations. If no toxicity is seen, an amendment will be submitted to increase the dose. M-LR will be given in sterile water.

The HSRP for arsenic is "Toxicokinetics of arsenicals." The cumulative dose of arsenic received by mice used in this study can be calculated from the intake of arsenic by mice which consume drinking water containing 5ppm inorganic arsenic. Based on a daily consumption of drinking water that contains 5ppm (ug/ml) of arsenic and a daily intake of 3ml of water per day, the daily dose is 15ug of arsenic. Based on a body weight of 0.025kg, the daily dosage is 0.6mg/kg and the cumulative 30-day dose is 18mg/kg.

NOTE ON DISPOSAL OF WASTE: A memo from **Exemption 6** received by email on March 30, 2015, outlined rules for the disposal of waste material produced by these studies. The regulated chemical is Sodium Arsenite (RCRA D004). Per the memo, waste sodium arsenite will be labeled Hazardous Waste and turned in to Chemical Services for disposal. Any drinking water which exceeds the 5.0mg/L(kg) arsenic limit will be labeled Hazardous Waste and turned in to Chemical Services for disposal. Any animal carcass containing a concentration at or about 5.0mg/kg arsenic will be managed as hazardous waste. It should be noted that it is unlikely that any mouse would attain a whole body concentration of

5mg/kg. Assuming mice are a) at steady state at the end of the 30-day exposure period, b) that 60% of the ingested dose of arsenic (administered as arsenite) is absorbed across the gastrointestinal tract, c) that drinking water contains 5ppm arsenic and daily intake is 3mL (15ug of arsenic ingested per day), and d) that the approximate whole body half-life of arsenic in the mouse is about 12 hours, then the calculated concentration of arsenic in a 25g mouse will be about 0.27mg/kg.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

Pharmaceutical grade materials are not available for either arsenic or M-LR..

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

na

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

na

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).

| NAME | ROLE | SPECIFIC RESPONSIBILITY | RELEVANT TRAINING |
|-------------|----------------------------------|---|---|
| Exemption 6 | Principal Investigator | Design of study, participation in dosing, weighing, monitoring and necropsy. | 40 years of experience in animal toxicology studies. All relevant animal use NHEERL training courses. |
| Exemption 6 | Associate Principal Investigator | Design of study, participation in dosing, weighing, monitoring and necropsy, recording of data. | Licensed veterinarian with 20 years experience. All relevant animal use NHEERL training courses. |
| Exemption 6 | Post-Doc | Participation in dosing, weighing, monitoring and necropsy, recording of data. | Seven years of experience using animals in research. All relevant animal use NHEERL training courses. |
| Exemption 6 | Student | Participation in dosing, | All relevant animal use NHEERL training |

| | | | | |
|--|--|------------------------|--|---|
| | | | weighing, monitoring and necropsy, recording of data. | courses. |
| Exemption 6 | | Principal Investigator | Design of study, participation in dosing, weighing, monitoring and necropsy. | >30 years of experience. All relevant animal use NHEERL training courses. |
| Exemption 6 Exemption 6 Exemption 6 | | Technical Staff | Participation in metabolism cage segment of the study. | >20 years of experience. All relevant animal use NHEERL training courses. |
| Exemption 6 | | Technical Staff | Participation in metabolism cage segment of the study | >20 years of experience. All relevant animal use NHEERL training courses. |
| Exemption 6 | | Technical Staff | Participation in metabolism cage segment of the study | >2 years of experience. All relevant animal use NHEERL training courses. |
| Exemption 6 | | Technical Staff | Participation in metabolism cage segment of the study | >2 years of experience. All relevant animal use NHEERL training courses. |
| | | | | . |
| | | | | |
| 1 | | Tech Support | Category C Procedures | All NHEERL required training is complete. |

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

- 1. Estimated number of breeding pairs and liveborn per year*** na
- 2. Breeding protocols and recordkeeping*** na
- 3. Methods for monitoring genetic stability*** na
- 4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR*** na

SECTION G - Euthanasia

- 1. When will the animals be euthanized relative to experimental procedures?***

Animals will be euthanized 24hrs after the dose of M-LR.

- 2. Describe the euthanasia techniques:***

Method(s): Euthanasia plus exsanguination

Agent(s): CO2

Dose (mg/kg): To effect

Volume:

Route: inhalation

Source(s) of information used to select the above agents/methods:

_ 2013 AVMA Guidelines on Euthanasia.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

na

4. Describe how death is to be confirmed.

Vital organ section, Prolonged absense of breathing.

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Euthanized as above

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

☒ Yes ☐ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.

2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.

3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.

4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.

5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.

6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.

7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.

8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

| | |
|----------------------------|------------------------------|
| EPA Principal Investigator | Certification Signature Date |
| Exemption 6 Exemption 6 | 03/31/2015 |

Submitted: 03/31/2015

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

| | | | | |
|--------------------------------|---------------|--------------|----------|-----------|
| Branch Chief/Division Director | Approval Date | Phone Number | Division | Mail Drop |
|--------------------------------|---------------|--------------|----------|-----------|

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| Exemption 6 | 03/31/2015 | Exemption 6 Lotus Notes Address Exemption 6 | TAD Branch DTB | MD Submitted to Branch Chief for Approval 03/31/2015 10:25 AM |
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ATTACHMENTS



18-04-001 PI resp.pdf



Exemption 6 LAPR Study Diagram 4-6-15.pptx



18-04-001 pre-screen.pdf

Actions

First Update notification sent: 03/09/2016
Second Update notification sent: 04/13/2016
First 2nd Annual notification sent:
03/02/2017
Second 2nd Annual notification sent:

1st Expiration notification sent: 03/02/2018
2nd Expiration notification sent:

History Log: